INTRACELLULAR HYDRION CONCENTRATION STUDIES.

I. The Relation of the Environment to the pH of Protoplasm and of its Inclusion Bodies.

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Recent micrurgical investigations (1, 2, 3) on the colorimetric determination of the protoplasmic pH have emphasized the need of studying the relation between the pH of the protoplasm of a living cell and that of its environment. Of the acids and bases which affect the pH of the environment some penetrate living cells while others apparently do not. This has been demonstrated by the change in color of cells stained with indicators. For example, with the use of neutral red it has been shown by previous investigators (4, 5) that living cells are readily permeable to CO_2 and NH_3 but not to HCl nor NaOH. This fact that the color of the intracellular stain can be readily shifted to the acid or the alkaline side suggests that the intraprotoplasmic pH can be changed very easily by environmental conditions, a conclusion which is at variance with experiments which indicate that protoplasm has a marked buffering power. Thus, when solutions of indicators, both in the acid and the alkaline states of their color ranges, are injected into living cells the colors quickly shift to those characteristic of a constant pH (6.9 ± 0.1). This has been found true for such varied types of cells as the ameba (1, 6), marine ova (2, 3), and various tissue cells of the frog and the mammal (6). In addition, there is the significant result that the localized increase in intraprotoplasmic acidity, caused by mechanical injury is almost immediately neutralized as long as no cytolysis results (1, 2, 3, 6).

In view of these facts it was considered advisable to test further the constancy of the intraprotoplasmic pH, to discover

whether this pH can be shifted appreciably without detriment to the cell and to obtain evidence, if any, of localized variations in the intracellular pH.

The purpose of the experiments described in this paper is to determine whether the intraprotoplasmic pH can be shifted by exposure to CO_2 or to NH_3 and whether the reaction to indicators of such intracellular structures as granules and vacuoles are comparable to those of the optically homogeneous protoplasmic matrix.

Before dealing with the actual experiments performed it is necessary to describe the manner in which the protoplasm becomes colored with neutral red and with the other dyes used. When cells are stained with neutral red or certain other basic dyes, the dye accumulates in or on the intracellular granules and vacuoles while the hyaline protoplasmic matrix remains colorless. This occurs not only when cells are stained by immersion in a solution of the dye but also when the dye is injected directly into the cell. In the latter case the color appears at first diffuse but gradually the granules and vacuoles take up more and more of the color until none of it can be detected in the hyaline cytoplasmic matrix. On the other hand the acid dyes used, *e.g.*, brom cresol purple, phenol red and cresol red, do not penetrate from the environment into the cells. When injected, however, they quickly spread through the cytoplasm giving to its hyaline matrix a more or less permanent and diffuse coloration (1, 2, 3, 6).

The fresh water *Amœba dubia* and the unfertilized eggs of the starfish, *Asterias forbesii*, and sanddollar, *Echinarachnius parma*, were used in these experiments. The amœba and the eggs were colored with the dyes either by the immersion method or by the microinjection method. Both methods were also used simultaneously on the same cell. The cells were then immersed in various acid and alkaline solutions and the color changes noted. For a study of the effect of NH_3 and CO_2 the cells were suspended in hanging drops of water from the roof of a special form of moist chamber which was closed except for narrow inlet and outlet tubes (7). The hanging drops were then charged with either CO_2 or with NH_3 by passing the moist gas through the chamber.

1. Effect of Acids and Bases on Amebæ Colored by the Injection of Acid Indicators Only.

Amebæ were injected with 0.4 per cent solution of brom cresol purple, phenol red and cresol red (8). These indicators were selected because they change color within the pH ranges tested (1, 3). Amebæ, injected with brom cresol purple, are uniformly blue (the alkaline range), with phenol red, a pale orange yellow (approaching the acid range). These findings accord with those already published (6) from which the pH of the freshwater ameba was placed at 6.9 ± 0.1.

Amebæ, colored by the injection of the above-mentioned dyes, were immersed in solutions of HCl (pH 5.5), NH_4Cl (pH 5.5), CO_2 charged water (pH 5.5), $NaHCO_3$ (pH 8), NH_4OH (pH 8) and NaOH (pH 8). The acidity of the first three solutions is sufficient to cause the indicators to take on the yellow color of their acid ranges, while the alkalinity of the last three solutions is sufficient to give to brom cresol purple the purple blue, and to phenol red and cresol red the bright red color of their alkaline ranges. It was found that the immersed amebæ all maintained their original colors as long as they remained alive. The color of those which rounded up and died changed to that characteristic for the pH of the environing medium.

These results indicate, either that there is no penetration of the acid or of the alkali from the solutions used, or that the protoplasm is sufficiently buffered to neutralize the acid or the alkali which does penetrate.

2. Effect of Acids and Bases on Cells Stained with Neutral Red and Injected with Acid Indicators.

a. Amœba dubia.

Since the permeability of cells to certain acids and bases can be demonstrated by the change in color of neutral red, amebæ were immersed in a solution of neutral red until various intracellular inclusions took on a red color. These amebæ were then injected with solutions of the indicators which color the cytoplasm diffusely. On immersing these doubly colored amebæ into the various acid and alkaline solutions the following results were obtained:

In accordance with the previous experiment it was found that immersion produced no change whatever in the diffuse coloration of the hyaline cytoplasmic matrix. On the other hand, the inclusion bodies which were stained with neutral red quickly became yellow in the solutions containing the NH_3 (NH_4OH and NH_4Cl) and bright red in those containing CO_2 ($NaHCO_3$ and CO_2 charged water).

These results imply that the pH of the hyaline cytoplasm does not change even when sufficient NH_3 or CO_2 penetrates to change the color of the intracellular inclusions. In other words, the pH of the intracellular inclusions can be shifted readily by the presence of CO_2 or of NH_3 in the environment while that of the protoplasmic matrix remains constant.

b. Unfertilized Eggs of the Sanddollar (Echinarachnius parma) and the Starfish (Asterias forbesii).

The protoplasm of these eggs is uniformly crowded with granules or macrosomes practically all of which ultimately stain a deep rose red with neutral red. The eggs were allowed to remain in sea-water containing neutral red only long enough to stain a small percentage of the granules. The eggs were then washed, transferred to hanging drops of sea-water in the moist chamber and injected with the indicator solutions. In the same chamber were placed, as controls, other hanging drops of sea-water colored with the same indicators. Ammonia gas was then passed through the chamber until the hanging drops became sufficiently saturated with ammonia to change the color of the control drops.

The color of the eggs was noted when the dyes in the control drops had assumed colors indicating a pH more alkaline than 8.4. In every case the color of the granules, stained with neutral red, changed from red (acid) to yellow (alkaline) while the diffuse coloration of the indicators in the hyaloplasm of the eggs persisted in registering the originally recorded pH of 6.8 ± 0.1 (3).

An experiment giving striking color contrasts is one in which three dyes, neutral red, phenol red and cresol red, were used for the purpose of detecting simultaneously the pH changes in the cytoplasm, the cytoplasmic granules, and the sea-water surrounding the eggs. It is to be remembered that neutral red

which stains the granules is red at a pH more acid than 6.8 and yellow at a pH more alkaline than 7.4. Phenol red which colors the hyaloplasm is yellow at a pH more acid than 6.8 and red at a pH more alkaline than 7.4, and cresol red which was used for the environing sea-water is yellow at a pH more acid than 7.8 and red at a pH more alkaline than 8.0. The experiment was the following: Eggs, stained with neutral red, were immersed in a hanging drop of sea-water colored with cresol red and were then injected with phenol red. The result was a striking picture of yellow eggs containing scattered red granules and surrounded by a medium of yellow sea-water. Ammonia gas was then passed through the chamber until the cresol red in the sea-water changed from yellow (acid) to red (alkaline). As soon as this occurred the cytoplasmic granules, stained with the neutral red turned yellow (alkaline) while the hyaloplasm maintained the original yellow (acid) color of the phenol red. The result was now a picture of uniformly yellow eggs standing out against a background of red sea-water. Carbonic acid gas was then passed through the chamber until it displaced the NH_3 in the hanging drops. As a result the original colors returned, viz., the sea-water again became yellow, the cytoplasmic granules turned from yellow to red but the cytoplasm itself remained yellow.

Since the cytoplasm has a pH of 6.8 ± 0.1 (3) which is in the acid range of phenol red the above experiment is not suited for detecting a possible effect of the CO_2 on the cytoplasmic pH. For this purpose it is necessary to use brom cresol purple (yellow at a pH more acid than 6.0 and blue at a pH more alkaline than 6.2) which, upon injection, colors the hyaloplasm blue. These eggs were immersed in a hanging drop of sea-water colored blue with the same dye. The hanging drop was suspended in the hermetic chamber through which moist CO_2 gas was made to stream until the sea-water became charged with CO_2 sufficiently to change its color from blue to yellow. The eggs in the yellow water kept their original blue color.

These experiments indicate that NH_3 and CO_2, both of which penetrate the protoplasm and affect the pH of the intracellular granules, do not shift the pH of the hyaloplasm as measured by the indicators.

3. Effect of CO_2 and of NH_3 on Amebæ whose Cytoplasm and Inclusion Bodies are Colored with the Same Indicator.

A possible error in the previous experiments lies in the fact that the coloration of the cytoplasmic inclusions and of the hyaline cytoplasm were not made with the same dye. For example, neutral red, which colors the cellular inclusions, is a basic dye, while the dyes used for producing a diffuse coloration are acidic. It is conceivable that this may be responsible for their difference in reaction to the penetrating CO_2 or NH_3.

To meet this objection it was found that methyl red could be used. Methyl red has already been used as a vital stain for plant protoplasm (9) and is a pH indicator, being red at a pH more acid than 5.0 and yellow at a pH more alkaline than 5.4. Immersion of amebæ in an aqueous solution of this dye stains the hyaline cytoplasm, its various inclusions and the nucleus an intense yellow. Amebæ colored in this way were placed in a moist chamber in hanging drops of the yellow aqueous solution of methyl red. Moist CO_2 gas was then passed through the chamber until the hanging drops turned from yellow to red. When this occurred it was found that the yellow stained inclusions of the ameba had also become red while the cytoplasm and nucleus remained yellow. Ammonia vapor was now passed through the chamber whereupon the color of the hanging drops and of the intracellular inclusions quickly changed back to yellow.

These experiments with methyl red clearly demonstrate the penetration of CO_2 into the living ameba [1] as registered by the change in color of the intracellular inclusions. The hyaline cytoplasm and the nucleus, however, maintain their original color and give no evidence of a change in pH.

[1] The neutral red method is not very favorable for detecting the penetration of CO_2 into cells since the granules stained with neutral red under normal conditions already have the rose red color characteristic for the acid range of the dye. On the other hand, methyl red under normal conditions stains the intracellular granules the yellow color of its alkaline range. Upon exposure to CO_2 the color of the granules changes to red, which is as decided an evidence for the penetration of the CO_2 as is the neutral red method for the penetration of NH_3.

4. The Effect of Penetrating Acids and Bases on the Nuclear pH.

The nuclei of immature starfish eggs were used in these experiments. The nuclei of different eggs were colored with cresol red, neutral red and phenol red by the microinjection method after which the eggs were exposed to CO_2 and to NH_3. In every case the color within the nuclei of living eggs remained constant irrespective of the color changes of the granules in the surrounding cytoplasm. In other words, the nucleus was found to be sufficiently buffered so that the intranuclear pH of 7.6–7.8 (3) remains unchanged. When the egg disintegrates by crushing or tearing, the nucleus undergoes changes (3) and loses all buffering action. The persisting spherical nuclear remnant is then immediately susceptible to acid and alkali changes in its environment.

Summary.

The presence of CO_2 or of NH_3 in the aqueous medium surrounding living cells readily changes the pH of the intracellular inclusions which stain with neutral red but does not change the pH of the protoplasmic matrix nor of the nucleus as long as the cell is alive.

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